J. Vet. Sci. (2006), **7**(3), 277–280

JOURNAL OF Veterinary Science

The 23S rRNA gene PCR-RFLP used for characterization of porcine intestinal spirochete isolates

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Using three reference strains of *Brachyspira hyodysenteriae* (B204, B234, B169), one *B. pilosicoli* (P43/6/78), one *B. murdochii* (56-150), one *B. intermedia* (PWS/A), one *B. innocens* (B256) and ten Korean isolates, PCR-RFLP analysis of DNA encoding 23S rRNA was performed to establish a rapid and accurate method for characterizing porcine intestinal spirochetes. Consequently, *B. hyodysenteriae* and *B. pilosicoli* revealed different restriction patterns; however, the other three species shared the same pattern. These findings are not consistent with a prior report. Differences in 23S rRNA gene sequences, between two *B. murdochii* strains, 56-150 and 155-20, were observed. These results indicate that 23S rRNA PCR-RFLP could be used as an identification method for pathogenic *Brachyspira* spp. (*B. hyodysenteriae* and *B. pilosicoli*) as well as an epidemiological tool for characterizing spirochetes isolated from swine.

Key words: *Brachyspira* spp., PCR-RFLP, 23S rRNA, spirochete

Introduction

Swine dysentery (SD) is a mucohaemorrhagic colitis of pigs caused by the anaerobic intestinal spirochete *Brachyspira hyodysenteriae*. Outbreaks of SD are relatively common in a number of developed and developing countries, especially where the use of antimicrobial agents is restricted [9]. Until recently, there were five species of Brachyspira identified from swine [20]. *B. innocens*, though it is similar to *B. hyodysenteriae*, is non-pathogenic and has been isolated from both healthy and sick pigs; this causes confusion in the diagnosis of SD [9]. *B. pilosicoli*, a weakly hemolytic spirochete, has been isolated from pigs with mucosal diarrhea [20]. *B. intermedia* is indole positive with weak hemolysis, and *B. murdochii* is, although morphologically similar to *B. hyodysenteriae*, weakly hemolytic and indole negative [13]. Many attempts have been made to characterize the porcine intestinal spirochete isolates by serological diagnosis [9], restriction endonuclease analysis (REA) [11,18], PCR [1,7,15], sequence analysis of genes [4,14], multilocus enzyme electrophoresis (MLEE) [10,12,13] and RAPD [5]. Recently, PCR-RFLP methods have been developed and used for 23S rRNA genes [3], *fla*A1 [8] and NADH oxidase (*nox*) [2,16,19]. In this study, the 23S rRNA gene PCR-RFLP was used for the characterization of Korean isolates.

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Materials and Methods

Microorganisms and DNA samples

Seven reference strains (Table 1) were used for the characterization of ten Korean isolates (National Veterinary Research and Quarantine Service, Korea), which were previously isolated from pigs with dysentery and identified using biochemical and serological methods [9,10,12,13]. Chromosomal DNA was prepared using the QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions.

PCR-RFLP

Using Gene Runner software (Hastings Software, USA), a 23S rRNA-specific primer set was designed and synthesized (Bionics, Korea). A forward primer, which corresponded to the *B. hyodysenteriae* 23S rRNA gene sequence (GenBank #U72699) between 999th to 1022th nucleotides and a reverse primer between 1492th to 1515th nucleotides were used to amplify a 517 bp PCR product. The PCR conditions consisted of 5 µl (50 ng/µl) of DNA and 1 µl each of primer (50 pM) in a 5 µl of 10× reaction buffer with 5 µl of 25 mM $MgCl_2$, 5 µl of 10 mM dNTP (each 2.5 mM) and 1 µl of 5 U Ex Taq DNA polymerase (TaKaRa, Japan) to a final volume of 50 µl on a thermal cycler (PTC-100; MJ Research, USA). PCR was initiated after an incubation step at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and

Table 1. Porcine intestinal spirochete species and strains

Species	Strain	23S rRNA gene	
		GenBank accession number	References
B. hyodysenteriae	B204,B234,B169	U72699	[21]
B. pilosicoli	P43/6/78	U72703	[21]
B. murdochii	56-150	AY748887 for partial sequence	[14]
B. intermedia	PWS/A	U72700	[18]
B. innocens	B256	U72701, U72702	[21]

Table 2. Predicted restriction fragments of 517 bp; DNA sequence of *Brachyspira* ribosomal DNA encoding 23S rRNA using different restriction endonucleases

Species	Predicted restriction fragments (bp)	
	*Taq*I	*Alu*I
B. hyodysenteriae	31* 41 63 **94**** **134** 154	40 72 168 237
B. pilosicoli	31* 41 **51** **166** 228	40 168 **309**
B. murdochii (155-20)	31* 41 63 154 228	**31*** 40 72 168 **206**
B. intermedia	31* 41 63 154 228	40 72 168 237
B. innocens	31* 41 63 154 228	40 72 168 237

*Values in underlined are fragments not visualized in 3 % agarose gels.
**Unique restriction fragments are shown in bold characters.

72°C for 30 s, with a final extension step at 72°C for 5 min. PCR products that were 517 bp were excised and purified from agarose gels using a Geneclean II Kit (Qbiogene, USA). Purified PCR products were digested with either *Taq*I or *Alu*I restriction enzymes (Promega, USA) according to the manufacturer's instructions. Digested fragments were visualized on 3% agarose gels. DNA sequencing reactions were performed on an automated DNA sequencer (ABI PRISM 3100 Genetic Analyzer; Applied Biosystems, USA). Sequencing data were assembled and edited using the BLAST method. Sequences were aligned and a phylogenic tree was constructed using DNAMAN (Lynnon BioSoft, Canada).

Results

Using a set of primers, the 517 bp PCR product was amplified from all reference strains and Korean isolates. Four PCR-RFLP patterns were predicted after digestion of PCR products with either *Taq*I or *Alu*I (Table 2). As expected, when digested with *Taq*I, unique 94, 134 bp fragments from *B. hyodysenteriae* and 51, 166 bp fragments from *B. pilosicoli* were produced (Fig. 1). The restriction enzyme *Alu*I produced a unique 309 bp fragment with *B. pilosicoli* (Fig. 2). However, a unique 206 bp fragment was not produced with *B. murdochii* (56-150). To better understand the genetic differences among spirochetes, a phylogenic tree was generated (Fig. 3).

Discussion

Intestinal spirochetes are frequently isolated from intestinal specimens of swine suffering from SD; however, they can also be isolated from healthy swine. The biochemical and morphological discrimination of different types of *Brachyspira* spp. is complicated by difficulties with culture techniques and common characteristics. Therefore, a rapid and accurate method is required for the accurate identification of pathogenic

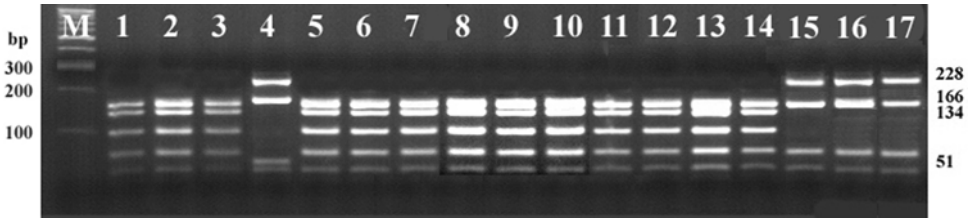

Fig. 1. PCR-RFLP fragments of the 23S rRNA gene in 3% agarose gel electrophoresis digestion with *Taq*I. Lane M: 100 bp DNA ladder; lane 1: *B. hyodysenteriae* B204; lane 2: *B. hyodysenteriae* B234; lane 3: *B. hyodysenteriae* B169; lane 4: *B. pilosicoli* P43/6/78; lane 5 to 14: *B. hyodysenteriae* field isolates; lane 15: *B. murdochii* 56-150; lane 16: *B. intermedia* PWS/A; lane 17: *B. innocens* B256.

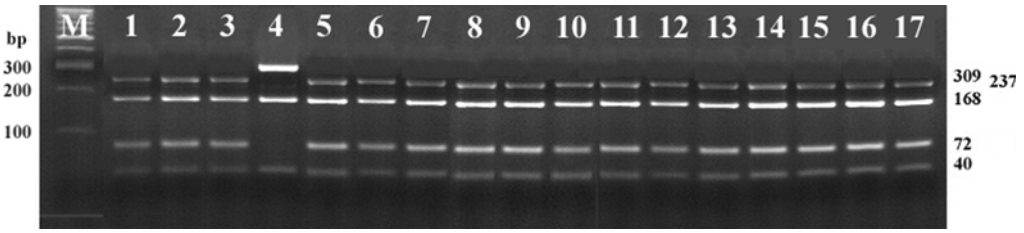

Fig. 2. PCR-RFLP fragments of the 23S rRNA gene in 3% agarose gel electrophoresis digestion with *Alu*I. Lane M: 100 bp DNA ladder; lane 1: *B. hyodysenteriae* B204; lane 2: *B. hyodysenteriae* B234; lane 3: *B. hyodysenteriae* B169; lane 4: *B. pilosicoli* P43/6/78; lane 5 to 14: *B. hyodysenteriae* field isolates; lane 15: *B. murdochii* 56-150; lane 16: *B. intermedia* PWS/A; lane 17: *B. innocens* B256.

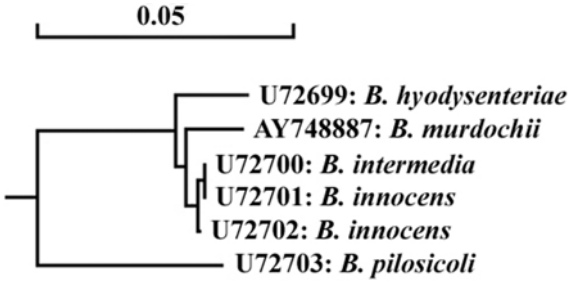

Fig. 3. Phylogenic analyses of 23S rRNA gene sequences in *Brachyspira* spp. The genetic distances are proportional to the relative sequence deviations between individual nucleotide sequences.

(*B. hyodysenteriae* and *B. pilosicoli*) differentiated from non-pathogenic porcine intestinal spirochetes. Historically, hemolysis patterns on blood agar, during primary isolation, have been used for general identification to distinguish pathogenic from non-pathogenic intestinal spirochetes [6]. However, there are some strains, which produce weak and/or intermediate hemolysis and can not be assigned to a specific group [3]. PCR-RFLP has been shown to produce accurate, rapid and reproducible results for the identification of porcine intestinal spirochetes [3,16,19]. For *nox*-based PCR-RFLP experiment [16], four sets of primers were used; this implies that the target sequences for primer binding are not highly conserved, though the restriction pattern was highly distinct. For a 16S rRNA PCR-RFLP experiment [17], it was not possible to differentiate *B. hyodysenteriae* from *B. intermedia*. However, 23S rRNA PCR-RFLP revealed a similar pattern within same species; the sequence for 23S rRNA was highly conserved among the same species [3].

Ten Korean isolates, previously classified as *B. hyodysenteriae* by biochemical and morphological methods, shared the same restriction pattern with *B. hyodysenteriae* reference strains (B204, B234, B169). The results for *B. pilosicoli*, *B. innocens* and *B. intermedia* were similar to those from a previous report [3]. According to a previous study, *B. murdochii* was expected to produce a unique 206 bp fragment [3]. However, the restriction pattern of *B. murdochii* (56-160) was the same as non-pathogenic intestinal spirochetes, which is a finding different from results previously reported [3]. Therefore, *B. murdochii* strains (155-20 [3] and 56-160) did not share the same restriction pattern, and 23S rRNA PCR-RFLP cannot be used for the discrimination of non-pathogenic intestinal spirochetes; though it can discriminate between pathogenic and non-pathogenic porcine intestinal spirochetes. In this study 3% agarose gels were used for the visualization of restriction fragments. The use of 12.5% polyacrylamide gels with silver nitrate staining for 23S rRNA PCR-RFLP is complicated by difficulties with handling and reading, and requires more time and effort. However, when using 3% agarose gel, the visualization of small DNA fragments produced during restriction enzyme treatment was very simple and produced clear results, which could be used for routine diagnosis.

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